



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

BArg448Lys polymorphism is associated with altered fibrin clot structure and fibrinolysis in type 2 diabetes

Citation for published version:

Greenhalgh, KA, Strachan, MW, Alzahrani, SH, Baxter, PD, Standeven, KF, Storey, RF, Ariens, RAS, Grant, PJ, Price, J & Ajjan, RA 2017, 'BArg448Lys polymorphism is associated with altered fibrin clot structure and fibrinolysis in type 2 diabetes', *Thrombosis and Haemostasis*, vol. 117, no. 2, pp. 295-302. <https://doi.org/10.1160/TH16-07-0554>

Digital Object Identifier (DOI):

[10.1160/TH16-07-0554](https://doi.org/10.1160/TH16-07-0554)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Thrombosis and Haemostasis

Publisher Rights Statement:

This is the author's peer-reviewed manuscript as accepted for publication.

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



B β Arg448Lys polymorphism is associated with altered fibrin clot structure and fibrinolysis in type 2 diabetes

Running title: B β Arg448Lys polymorphism in type 2 diabetes

K A Greenhalgh*, M W Strachan[†], S Alzahrani[‡], P D Baxter*, K F Standeven*, R F Storey[#],
R A S Ariens*, P J Grant*, J F Price^{§*} & R A Ajjan^{**}

*Division of Cardiovascular and Diabetes Research, Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds, LS2 9JT, UK.

[†]Metabolic Unit, Western General Hospital, Edinburgh, UK.

[‡]Specialised Diabetes and Endocrine Centre, King Fahad Medical City, Riyadh, KSA

[#]Department of Infection, Immunity & Cardiovascular Disease, University of Sheffield, Sheffield, UK

[§]Centre for Population Health Sciences, University of Edinburgh, Edinburgh, UK.

* JFP and RAA contributed equally to this work

Text word count (excluding tables, figure legends and references): 3,4269

Abstract word count: 250

Corresponding Author: R. A. Ajjan, Division of Cardiovascular and Diabetes Research, Leeds Institute of Cardiovascular and Metabolic Medicine, Clarendon Way, University of Leeds, Leeds LS2 9JT, UK. Phone: +44-113-343 7720, fax: +44-113-343 7738, email: r.ajjan@leeds.ac.uk

This work was funded by the Medical Research Council and the British Heart Foundation.

Conflict of Interest Disclosure Statement: The authors have nothing to disclose in relation to this work.

All authors have had full access to data and contributed to drafting of the paper.

Abstract

Background

Both type 2 diabetes (T2DM) and B β 448Lys variant of fibrinogen are associated with dense fibrin clots, impaired fibrinolysis and increased cardiovascular risk.

Objectives

Investigate whether B β Arg448Lys adds to vascular risk by modulating fibrin network structure and/or fibrinolysis in diabetes. The primary aim was to study effects of B β Arg448Lys on fibrin network characteristics in T2DM. Secondary aims investigated interactions between gender and B β Arg448Lys substitution in relation to fibrin clot properties and vascular disease.

Patients/Methods

Genotyping for B β Arg448Lys and dynamic clot studies were carried out on 822 T2DM patients enrolled in the Edinburgh Type 2 Diabetes Study. Turbidimetric assays of individual plasma samples analysed fibrin clot characteristics with additional experiments conducted on clots made from purified fibrinogen, further examined by confocal and electron microscopy.

Results

Plasma clot lysis time in B β 448Lys was longer than B β 448Arg variant (mean \pm SD; 763 \pm 322 and 719 \pm 351 s, respectively; $p<0.05$). Clots made from plasma-purified fibrinogen of individuals with Arg/Arg, Arg/Lys and Lys/Lys genotypes showed differences in fibre thickness (46.75 \pm 8.07, 38.40 \pm 6.04 and 25 \pm 4.99 nm, respectively; $p<0.001$) and clot lysis time (419 \pm 64, 442 \pm 87 and 517 \pm 65 s, respectively; $p=0.02$), directly implicating the polymorphism in the observed changes. Women with B β 448Lys genotype had increased risk of cerebrovascular events and were younger compared with B β 448Arg variant (67.2 \pm 4.0 and 68.2 \pm 4.4 years, respectively; $p=0.035$).

Conclusions

Fibrinogen B β 448Lys variant is associated with thrombotic fibrin clots in diabetes independently of traditional risk factors. Prospective studies are warranted to fully

understand the role of B β Arg448Lys in predisposition to vascular ischaemia in T2DM with the potential to develop individualised antithrombotic management strategies.

Keywords: Diabetes Mellitus, Fibrinogen, Genetic Polymorphism, Thrombosis, Vascular Diseases

<p>What is known on this topic</p>	<ul style="list-style-type: none"> • The common genetic variant of fibrinogen BβArg448Lys is associated with significant changes in clot structure and fibrinolysis. • Diabetes is associated with qualitative and quantitative changes in coagulation proteins, resulting in dense fibrin networks that are difficult to lyse.
<p>What this paper adds</p>	<ul style="list-style-type: none"> • We demonstrate that Bβ448Lys variant of fibrinogen is associated with thrombotic fibrin networks in a highly complex and multifactorial condition such as type 2 diabetes. • Our data also suggest an adverse clinical vascular effect of this polymorphism in diabetes, which may be gender specific. • Further clinical studies are needed to investigate the role of this common polymorphism in vascular risk in diabetes, which may lead to individualised therapies aiming to reduce cardiovascular events, the main cause of mortality in this population.

Introduction

Despite advances in medical therapy, cardiovascular complications remain the main cause of mortality in diabetes. In addition to increased risk of a first atherothrombotic event, prognosis following vascular ischaemia remains poor, in contrast to individuals without diabetes, in whom clinical outcome has improved over the past decade (1-7). Current evidence indicates that the adverse clinical outcome in diabetes following vascular ischaemia is due to more extensive vascular pathology, increased thrombosis potential and high prevalence of heart failure, the latter being partly related to atherothrombotic complications (1-4;8).

The prothrombotic environment in diabetes plays a key role in increased cardiovascular risk in this population. Formation of an obstructive thrombus in a diseased vessel occurs secondary to complex interactions between the cellular and protein phase of coagulation (reviewed in (5)). The thrombus, or blood clot, is composed of a backbone of fibrin fibres, with embedded platelets and blood cells. Diabetes is associated with qualitative and quantitative changes in procoagulant and anti-fibrinolytic proteins leading to fibrin clots with tight network structure and resistance to lysis, a structure that has been associated with increased cardiovascular risk (6;7;9-17). Moreover, we have recently shown gender differences in the association of fibrin network structure with metabolic and cardiovascular factors, suggesting a differential fibrin-related thrombosis risk in men and women with diabetes (18).

Fibrinogen B β Arg448Lys, a common genetic polymorphism of which the Lys448 allele has a frequency of 15% to 20% in whites, is associated with both increased vascular risk and hypofibrinolysis (9;19-22). Using recombinant techniques, we have previously shown that clots made from the B β 448Lys variant of fibrinogen have a compact structure of thin fibres and small pores, with increased stiffness and greater resistance to lysis (9), consequently predisposing to a thrombotic environment.

Taken together, both diabetes and B β Arg448Lys variants of fibrinogen induce changes in fibrin networks that are associated with increased cardiovascular risk. Despite

the relatively high prevalence of diabetes and B β Arg448Lys polymorphism, the effect of B β Arg448Lys on clot structure in diabetes remains unknown. Therefore, the primary aim of this study was to identify whether B β Arg448Lys has an independent effect on the fibrin network in individuals with type 2 diabetes (T2DM). Our secondary aims included the analysis of potential gender differences of B β 448Arg to Lys substitution on clot characteristics and the preliminary investigation of the clinical vascular effect of this polymorphism in diabetes.

Materials and Methods

Study population and genotyping

The study protocol of the Edinburgh Type 2 Diabetes Study (ET2DM) has been described elsewhere (23). Briefly, 1,066 patients with T2DM (age 60–75 years) were recruited from a population-based register including patients treated in both primary and secondary care after informed consent. The study population has been shown previously to be largely representative of all those randomly selected to participate (N=5,454). A total of 822 plasma samples were available for genotyping and clot structure analysis.

Blood samples were taken into tubes containing 1ml 0.109M tri-sodium citrate anticoagulant to a final volume of 10ml without a tourniquet after a 4-hour fast, usually at midday. The first 5ml were used for clinical tests and platelet-poor plasma was separated from subsequent samples. Blood was centrifuged at 2,400g for 20 minutes at room temperature and plasma was aspirated. DNA was purified from blood samples and genotyping for B β Arg448Lys variants of fibrinogen was performed as previously described (24).

Analysis of fibrinogen levels, plasma clot formation and lysis

Fibrinogen levels and fibrin clot characteristics were measured in the 822 plasma samples, as previously described (18). Plasma levels of fibrinogen were studied by Clauss assay. Clot structure/fibrinolysis was assessed by turbidimetric assays.

Fibrinogen purification

Plasma samples were chosen for each variant (Arg/Arg, Arg/Lys and Lys/Lys; n=12 samples for each group), which were matched for sex and glycaemic control, measured as HbA1C, and pooled. Fibrinogen was purified by affinity chromatography using a calcium-dependent IF-1 monoclonal antibody and an automated chromatography system, as described (9). Samples were dialysed against 100mM NaCl, 50mM Tris, pH 7.4 using

dialysis cellulose membrane tubing, size 10 x 6mm, for 2 hours and subsequently overnight. Integrity and purity of plasma-purified fibrinogen were analysed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). Fibrinogen concentration was determined and adjusted to a dilution of 1 mg/ml. Four replicates were performed for each variant and experiments were repeated on three different occasions. Results are presented as mean±standard deviation (SD).

Analysis of clot formation and lysis in a purified system

A total of 50µl of purified fibrinogen was mixed with 50µl lysis mix (FXIII [ZLB Behring] and plasminogen [ERL] in 100mM NaCl, 50mM Tris, pH 7.4). Polymerisation and lysis were initiated by the addition of 50µl clotting mix (thrombin [Millipore] and tissue plasminogen activator [Technoclone] in 7.5mM CaCl₂, 100mM NaCl, 50mM Tris, pH 7.4), at ambient temperature in a 96-well plate. Final concentrations in the fibrinogen, clotting and lysis mix were 333µg/ml fibrinogen, 22µg/ml FXIII, 25µg/ml plasminogen 0.5U/ml thrombin and 0.312µg/ml tissue plasminogen activator. Four replicates were performed for each variant and experiments were repeated on 3 different occasions. Increase in turbidity at 340nm was continuously monitored every 12 seconds on an EL_x-808 IU ultramicroplate reader over a period of 120 minutes.

Laser scanning confocal microscopy

Plasma purified fibrinogen (30 µl) was used in the presence of 0.025mg/ml 488 fluorescent-labelled fibrinogen. Clotting was initiated with 5µl of activation mix (containing thrombin in 35mM CaCl₂, 50mM Tris, 100mM NaCl, pH 7.4), to the fibrinogen solution. Final concentrations in the clotting mix were 400µg/ml fibrinogen and 0.05U/ml thrombin. Subsequently, 30µl of this mixture was pipetted into Ibidi slides in duplicate. Clots were visualised using Leica TCS SP-2 laser scanning confocal equipment on an inverted DM IRE 2 microscope with 40x/1.3 oil pH 3 objective lens. At least three areas of 512 x 512 pixels were characterised per sample.

Electron Microscopy

Purified fibrinogen (45µl) was clotted upon addition of 5µl of activation mix (containing thrombin in 2.5mM CaCl₂, 50mM Tris, 100mM NaCl, pH 7.4), to the fibrinogen solution in specially devised small, perforated plastic vessels. Final concentrations in the clotting mix were 450µg/ml fibrinogen and 0.5U/ml thrombin. Plasma was diluted 1:2 in 50mM Tris, 100mM NaCl, pH7.4 and clotted with 5µl 0.05M CaCl₂, 11U/ml thrombin in 50mM Tris, 100mM NaCl, pH 7.4. Samples were incubated at room temperature in a moist chamber for 2 hours, washed with sodium cacodylate buffer (0.078M cacodylic acid, pH 7.4) and subsequently fixed overnight in 2% glutaraldehyde. Clots were recovered and further processed by a stepwise dehydration with an acetone gradient and sputter coated with platinum palladium in a 208 HR high-resolution sputter coater. Samples were viewed and photographed using a field-emission scanning electron microscope (LEO1530 FEGSEM, Leo Electron Microscopy) in 3 different areas of each clot. Images were captured using Leo 32 version 03.0210 software and cropped using Paintshop Pro version 8.0. Fibre diameters of all clots were measured with image analysis software package ImageJ 1,23y (National Institutes of Health).

Statistical analysis and power calculations

Exploratory analysis was done using SPSS program version 16. Between-group comparisons of normally distributed variables were carried out using t-test, non-normally distributed variables were log-transformed before a t-test was carried out. For plasma purified samples, where three genotypes were compared, an ANOVA was carried out. Post-hoc t-tests were then used to report p-values between two groups. The R environment for statistical computing was used to fit logistic regression models to identify associations between BβArg448Lys polymorphism and vascular disease. The effect of predictors in the regression model was characterised using 95% confidence intervals for the odds ratio coefficients.

Previous work has shown that 10% prolongation in clot lysis time is associated with atherothrombotic conditions (18;25;26). To detect a 10% increase in clot lysis time with B β 448Lys variants of fibrinogen, 181 samples were required given the standard deviation of the response variable in our cohort of 56% (at $p < 0.05$ with a power of 80%). Given that the frequency of B β 448Lys variant of fibrinogen in our population of 31%, a total of 790 samples were required for analysis. Therefore, our cohort of 822 patients has enough power to detect a 10% increase in clot lysis time with B β 448Lys variant of fibrinogen.

Results

Patient characteristics

Clinical characteristics of patients are described in Table I. The distribution of the three allelic fibrinogen variants was as expected; the majority of patients had the Arg/Arg variant (n=564), with fewer patients (n=234) having the Arg/Lys, and 24 patients possessing the Lys/Lys variant.

Cardiometabolic risk factors, including blood pressure, body-mass index, waist circumference, waist/hip ratio, HbA1c and lipid profile were similar in individuals with the two variants of fibrinogen. Treatment was also similar when comparing individuals with B β 448Arg and B β 448Lys genotypes of fibrinogen (summarised in Table I).

B β Arg448Lys and plasma-derived clots

B β 448Lys is associated with changes in clot structure and fibrinolysis

Clot maximum absorbance in carriers of B β 448Lys was higher than B β 448Arg (0.37 ± 0.10 and 0.35 ± 0.10 arbitrary units (au), respectively; $p=0.03$) and lysis time longer (763 ± 322 and 719 ± 351 s, respectively; $p=0.01$). There was no significant difference in lag time between B β 448Arg and B β 448Lys plasma (528 ± 125 and 523 ± 116 s, respectively; $p=0.70$). Results are summarised in Figure 1A–C.

B β 448Lys is associated with higher fibrinogen levels than B β 448Arg

Fibrinogen levels were higher in individuals with B β 448Lys variant when compared with B β 448Arg over the whole group (3.70 ± 0.74 and 3.59 ± 0.73 mg/ml, respectively; $p=0.03$). When split by gender, this difference was only significant in women at 3.81 ± 0.70 and 3.70 ± 0.70 mg/ml for B β 448Lys and B β 448Arg, respectively; $p=0.01$), with no difference detected in males (3.52 ± 0.69 and 3.49 ± 0.73 mg/ml, respectively; $p=0.67$).

The differences in lysis time and maximum absorbance between the two variants were still significant after adjusting for fibrinogen levels in the whole group ($p=0.03$ and 0.009

respectively). Therefore, the differences in lysis time and clot density are unlikely to be due to altered fibrinogen levels comparing the two variants of fibrinogen.

B β Arg448Lys and fibrinogen-derived clots

B β 448Lys is associated with decreased maximum turbidity and impaired lysis

To further investigate the effects of the polymorphism on clot structure/lysis in the absence of other plasma proteins, and in order to control for fibrinogen plasma levels, we undertook turbidity and lysis analysis of clots made from pools of Arg/Arg, Arg/Lys and Lys/Lys variants of fibrinogen. Purity and integrity of fibrinogen preparations prior to experiments were confirmed by SDS-PAGE.

Maximum absorbance. Clot maximum absorbance significantly differed between Arg/Arg, Arg/Lys and Lys/Lys fibrinogen variants ($p=0.003$). Clot maximum absorbance of Arg/Arg fibrinogen was significantly greater than Arg/Lys or Lys/Lys fibrinogen (0.0357 ± 0.007 , 0.0321 ± 0.005 and 0.0297 ± 0.004 au, respectively; $p=0.05$ and 0.001 respectively). Although maximum turbidity of clots made from Arg/Lys variant appeared greater than that of Lys/Lys fibrinogen, the difference failed to reach statistical significance ($p=0.09$; Figure 2A).

Fibrinolysis. Lysis time increased in a stepwise manner in clots made from Arg/Arg, Arg/Lys and Lys/Lys variants (419 ± 64 , 442 ± 87 and 517 ± 65 s, respectively, $p=0.02$). This difference was only significant between Arg/Arg and Lys/Lys fibrinogen ($p=0.003$), and Arg/Lys and Lys/Lys ($p=0.05$) as shown in Figure 2B.

B β 448Lys is associated with compact clots and thinner fibrin fibres

Confocal microscopy. Fibrin networks in hydrated clots made from purified Lys/Lys fibrinogen were more compact than those of Arg/Lys or Arg/Arg fibrinogen (Figure 3A). Clot density was further assessed using an in-house developed computer macro. Fibrin network density significantly differed between Arg/Arg, Arg/Lys and Lys/Lys fibrinogen variants

($p < 0.001$). Fibrin network made from purified Arg/Arg fibrinogen was less dense compared with Arg/Lys or Lys/Lys (104 ± 31 , 496 ± 60 and 434 ± 50 au, respectively; $p < 0.001$). There was no significant difference in clot density between Arg/Lys and Lys/Lys ($p = 0.2$).

Scanning electron microscopy. This technique was employed to study clot ultrastructure and fibrin fibre thickness. The more compact clot structure in the presence of Lys allele was confirmed, which was also associated with thinner fibres. Fibre thickness significantly differed between Arg/Arg, Arg/Lys and Lys/Lys fibrinogen variants ($p < 0.001$). Fibre thickness of clots made from purified Arg/Arg, Arg/Lys and Lys/Lys variants was 46.75 ± 8.07 , 38.40 ± 6.04 and 25 ± 4.99 nm, respectively; $p < 0.001$ between all variants (Figure 3B,C) consistent with the turbidimetric data presented above (Figure 2A). Similar observations were made for plasma samples; fibre thickness of clots made from plasma Arg/Arg, Arg/Lys and Lys/Lys variants was 49.76 ± 8.19 , 32.08 ± 6.21 and 24.04 ± 6.38 nm, respectively; $p < 0.001$ by ANOVA and between all variants.

The clinical role of B β Arg448Lys variants of fibrinogen

B β Arg448Lys and age

Mean age of subjects with B β 448Lys was lower compared with B β 448Arg across the whole group (67.6 ± 4.1 and 68.3 ± 4.2 years, respectively; $p = 0.02$). This age difference was only significant in women (67.2 ± 4.0 and 68.2 ± 4.4 years, respectively; $p = 0.04$) and not men (67.9 ± 4.23 and 68.4 ± 4.0 years, respectively; $p = 0.34$). Results are summarised in Figure 4. There was no significant difference in age between males and females across the whole group (68.2 ± 4.1 and 67.9 ± 4.3 years, respectively; $p = 0.24$).

B β Arg448Lys and history of vascular ischaemia

In the whole group, the B β 448Lys variant was associated with previous cerebrovascular incidents and transient ischaemic attacks (odds ratio [OR], 95% confidence interval [CI] 1.87, 1.13–3.11; $p = 0.015$; $n = 822$). This value was evident even after adjusting for traditional risk

factors, including total cholesterol, high-density lipoprotein, cholesterol, HbA1c, blood pressure and smoking (OR, 95% CI 1.98, 1.16–3.37; $p=0.016$). When the group was split by gender, the association of B β 448Lys variant for stroke/transient ischaemic attack was only significant in females and not males, whether adjusted (OR, 95% CI 4.18, 1.56–11.22; $p=0.003$ female: OR, 95% CI 1.43, 0.74–2.76; $p=0.27$ male) or unadjusted (OR, 95% CI 3.69, 1.49–9.13; $p=0.0045$ female: OR, 95% CI 1.38, 0.73–2.62; $p=0.32$ male). Data are summarised in Figure 5.

No association between B β Arg448Lys polymorphism and a history of coronary artery disease or myocardial infarction was detected in the whole population or when men and women were analysed separately.

B β Arg448Lys and current vascular pathology

Carotid intima media thickness was similar in carriers of B β 448Arg and B β 448Lys variants (1.00 ± 0.23 and 1.04 ± 0.32 mm, respectively; $p=0.08$), as was ankle-brachial index (ABI) (1.00 ± 0.23 and 0.97 ± 0.32 , respectively; $p=0.09$).

When analysed by gender, intra mural thickness (IMT) was similar in carriers of B β 448Arg and B β 448Lys in males (1.00 ± 0.34 and 1.05 ± 0.34 mm, respectively; $p=0.14$) and females (1.01 ± 0.33 and 1.04 ± 0.23 mm respectively; $p=0.30$). ABI was similar in carriers of B β 448Arg and B β 448Lys in males (1.00 ± 0.17 and 0.98 ± 0.228 , respectively; $p=0.84$) but a difference was detected in females (1.01 ± 0.16 and 0.95 ± 0.23 , respectively; $p=0.03$).

Discussion

Diabetes, a prothrombotic condition, is associated with compact fibrin networks and resistance to fibrinolysis, which contribute to the increased risk of vascular events in this population. Our work is the first study to investigate the functional role of B β Arg448Lys variants of fibrinogen in individuals with T2DM and we demonstrate an additional effect of this polymorphism on clot structure and fibrinolysis.

There are a number of novel observations emerging from this work in relation to individuals with T2DM: i) carriers of B β 448Lys variant of fibrinogen display compact plasma clots and impaired fibrinolysis compared with B β 448Arg, ii) in a purified system, B β 448Lys is associated with thinner fibrin fibres and trends in impaired fibrinolysis compared with B β 448Arg, supporting a direct role of this polymorphism in changes observed with plasma clots, iii) an association between B β 448Lys variant of fibrinogen and plasma protein level was evident but only in women, and iv) carriers of B β 448Lys variant were younger, suggesting early mortality in this group, which may be gender-specific and attributable to increased risk of vascular events.

An inherent difficulty in studying the functional effects of B β 448Lys variant on clot structure and lysis is the association of this polymorphism with plasma fibrinogen levels (20). This is believed to be related to linkage disequilibrium [of the -448G/A polymorphism](#) with [-455 G/A and -148 C/T polymorphisms](#) [B \$\beta\$ Gly455Ala and B \$\beta\$ Cys148Thr variants of fibrinogen of the \$\beta\$ fibrinogen gene](#), [polymorphisms that](#) determine protein levels, lying in the 5' flanking region and promoter region of the β -chain of fibrinogen, respectively. In the present work, the association between B β 448Lys and fibrinogen levels was only evident in women, indicating that environmental factors have stronger effects on fibrinogen levels than the B β Arg448Lys polymorphism in men with T2DM. Therefore, the genetic influence on thrombosis risk can differ in men and women with T2DM, which may have future therapeutic implications.

When plasma samples were studied, clot density was higher and lysis time longer in individuals with B β 448Lys compared with B β 448Arg variant. Although fibrinogen levels were

higher in carriers of B β 448Lys, correction for protein levels did not affect the role of the polymorphism on clot density or lysis time, suggesting a direct effect of the polymorphism on fibrin structure and fibrinolysis. To confirm that the observed changes in clot structure/lysis with B β 448Lys variant of fibrinogen are not related to altered plasma protein levels, we have undertaken experiments in a purified system and have shown that clot maximum absorbance decreases with increasing number of copies of the B β 448Lys allele. This further supports the role of this polymorphism in altered clot structure in individuals with T2DM. These turbidimetric data were reinforced by confocal and electron microscopy, showing increasing density of the fibrin clot and decreasing fibre thickness with increasing copies of the B β 448Lys allele. Previous work using a recombinant system has shown thinner fibrin fibres with B β 448Lys variant of fibrinogen, but it was unclear whether carrying one allele results in an intermediate phenotype, which is demonstrated in this study. Collectively, these results confirm the observed changes in plasma clot structure are independent of fibrinogen levels and that the genetic variant of fibrinogen studied has an additive effect on the fibrin network in individuals with T2DM. Although these findings have potential clinical implications, a further limitation of assessing plasma fibrin networks *ex vivo* to be acknowledged is that it ignores the potential influence of platelets, endothelial, or smooth muscle cells on clot properties.

Despite similarities in clot structure using purified and recombinant proteins, clot lysis outside the plasma environment showed some inconsistencies. In our previous study, using recombinant protein, the difference in clot lysis between the two variants was only evident in the plasma environment (9). In contrast, our current study shows differences in clot lysis made from the different variants of fibrinogen in the absence of plasma proteins. There are a number of possible explanations for this discrepancy. The current work used purified fibrinogen, which may have been contaminated with small quantities of plasma proteins not detected by SDS-PAGE. Another explanation is related to diabetes-induced post-translational modifications in fibrinogen that affect clot structure and fibrinolysis (7;27). A differential glycation, or other modification such as oxidation, may have occurred in the two

variants, resulting in altered clot lysis, particularly as lysine residues are amenable to such modifications (28).

One observation from our work suggests that the B β 448Lys variant is associated with adverse clinical outcome, as individuals with B β 448Lys variant were significantly younger than those with the B β 448Arg variant, which may indicate premature mortality in this population. Interestingly, the younger age of carriers of B β 448Lys variant only applied to women, who also demonstrated an increased risk of cerebrovascular events in carriers of this polymorphism. This further suggests a gender difference in the interaction between the polymorphism and fibrin-related thrombosis risk in individuals with T2DM. Similar gender-specific association between stroke and B β 448Lys variant of fibrinogen has been documented in a non-diabetic population before (20) and this therefore requires further investigation. However, we should be cautious in our interpretations as the study was not powered to investigate a clinical effect of this polymorphism. Whilst the difference in age was statistically significant, the potential survival benefit with the Arg448 variant is relatively modest and future research is needed to clarify whether this polymorphism has a more deleterious effect in particular subgroups of patients with diabetes. These findings are therefore merely hypothesis-generating and require further investigation in large longitudinal studies. We have further identified differences in ABI, a measure of peripheral vascular disease, and B β Arg448Lys genetic variants of fibrinogen, which was again only evident in women. The presence of peripheral vascular disease indicates extensive vascular pathology, supporting an association of B β 448Lys with severity of vascular pathology. However, caution should be exercised when interpreting these data, as clinical relevance of the documented differences can only be accurately assessed in a longitudinal study that is currently ongoing.

In summary, the current work demonstrates that the common genetic variant of fibrinogen B β 448Lys has an additive effect over that of T2DM on fibrin clot structure and

fibrinolysis. Moreover, our data suggest an interaction between B β Arg448Lys variants of fibrinogen, gender and clinical vascular disease that requires further investigation. Overall, this study indicates an important role for fibrinogen gene variants in determining thrombosis risk even in a complex multifactorial condition such as T2DM. Further clinical studies are needed to investigate the role of this common polymorphism in vascular risk in diabetes, which may lead to individualised therapies to reduce cardiovascular events, the main cause of mortality in this population.

Acknowledgements:

K. A. Greenhalgh researched data and wrote the manuscript. M. W. Strachan reviewed/edited the manuscript. S. Alzahrani researched data. P. D. Baxter researched data and reviewed the manuscript. K. Standeven reviewed the manuscript. R Storey reviewed/edited the manuscript, R .A. S Ariens reviewed/edited the manuscript. P. J. Grant reviewed the manuscript. J. F. Price wrote/reviewed/edited the manuscript. R. A. Ajjan researched data and wrote/reviewed/edited the manuscript.

The authors would like to acknowledge the help of Fladia Phoenix with laboratory assistance.

Reference List

- (1) Puri R, Kataoka Y, Uno K, et al. The distinctive nature of atherosclerotic vascular disease in diabetes: pathophysiological and morphological insights. *Curr Diab Rep* 2012; 12: 280–285.
- (2) Ferreiro JL, Gomez-Hospital JA, Angiolillo DJ. Platelet abnormalities in diabetes mellitus. *Diab Vasc Dis Res* 2010; 7: 251–259.
- (3) Alzahrani SH, Ajjan RA. Coagulation and fibrinolysis in diabetes. *Diab Vasc Dis Res* 2010; 7: 260–273.
- (4) Mercer BN, Morais S, Cubbon RM, et al. Diabetes mellitus and the heart. *Int J Clin Pract* 2012; 66: 640–647.
- (5) Ajjan R, Grant PJ. Coagulation and atherothrombotic disease. *Atherosclerosis* 2006; 186: 240–259.
- (6) Kannel WB, D'Agostino RB, Wilson PW, et al. Diabetes, fibrinogen, and risk of cardiovascular disease: the Framingham experience. *Am Heart J* 1990; 120: 672–676.
- (7) Pieters M, van Zyl DG, Rheeder P, et al. Glycation of fibrinogen in uncontrolled diabetic patients and the effects of glycaemic control on fibrinogen glycation. *Thromb Res* 2007; 120: 439–446.
- (8) Hess K, Alzahrani SH, Mathai M, et al. A novel mechanism for hypofibrinolysis in diabetes: the role of complement C3. *Diabetologia* 2012; 55: 1103–1113.
- (9) Ajjan R, Lim BC, Standeven KF, et al. Common variation in the C-terminal region of the fibrinogen beta-chain: effects on fibrin structure, fibrinolysis and clot rigidity. *Blood* 2008; 111: 643–650.
- (10) Jorneskog G, Egberg N, Fagrell B et al. Altered properties of the fibrin gel structure in patients with IDDM. *Diabetologia* 1996; 39: 1519–1523.
- (11) Raynaud E, Perez-Martin A, Brun J, et al. Relationships between fibrinogen and insulin resistance. *Atherosclerosis* 2000; 150: 365–370.

- (12) Diamant M, Nieuwland R, Pablo RF, et al. Elevated numbers of tissue-factor exposing microparticles correlate with components of the metabolic syndrome in uncomplicated type 2 diabetes mellitus. *Circulation* 2002; 106: 2442–2447.
- (13) Vaidyula VR, Rao AK, Mozzoli M, et al. Effects of hyperglycemia and hyperinsulinemia on circulating tissue factor procoagulant activity and platelet CD40 ligand. *Diabetes* 2006; 55: 202–208.
- (14) Mansfield MW, Heywood DM, Grant PJ. Circulating levels of factor VII, fibrinogen, and von Willebrand factor and features of insulin resistance in first-degree relatives of patients with NIDDM. *Circulation* 1996; 94: 2171–2176.
- (15) Ceriello A, Esposito K, Ihnat M, et al. Simultaneous control of hyperglycemia and oxidative stress normalizes enhanced thrombin generation in type 1 diabetes. *J Thromb Haemost* 2009; 7: 1228–1230.
- (16) Dunn EJ, Philippou H, Ariens RA, et al. Molecular mechanisms involved in the resistance of fibrin to clot lysis by plasmin in subjects with type 2 diabetes mellitus. *Diabetologia* 2006; 49: 1071–1080.
- (17) Watala C, Pluta J, Golanski J, et al. Increased protein glycation in diabetes mellitus is associated with decreased aspirin-mediated protein acetylation and reduced sensitivity of blood platelets to aspirin. *J Mol Med (Berl)* 2005; 83: 148–158.
- (18) Alzahrani SH, Hess K, Price JF, et al. Gender-specific alterations in fibrin structure function in type 2 diabetes: associations with cardiometabolic and vascular markers. *J Clin Endocrinol Metab* 2012; 97: E2282–E2287.
- (19) French JK, Van de Water NS, Sutton TM, et al. Potential thrombophilic mutations/polymorphisms in patients with no flow-limiting stenosis after myocardial infarction. *Am Heart J* 2003; 145: 118–124.
- (20) Carter AM, Catto AJ, Bamford JM, et al. Gender-specific associations of the fibrinogen B beta 448 polymorphism, fibrinogen levels, and acute cerebrovascular disease. *Arterioscler Thromb Vasc Biol* 1997; 17: 589–594.

- (21) Behague I, Poirier O, Nicaud V, et al. Beta fibrinogen gene polymorphisms are associated with plasma fibrinogen and coronary artery disease in patients with myocardial infarction. The ECTIM Study. Etude Cas-Temoins sur l'Infarctus du Myocarde. *Circulation* 1996; 93: 440–449.
- (22) Jacquemin B, Antoniades C, Nyberg F, et al. Common genetic polymorphisms and haplotypes of fibrinogen alpha, beta, and gamma chains affect fibrinogen levels and the response to proinflammatory stimulation in myocardial infarction survivors: the AIRGENE study. *J Am Coll Cardiol* 2008; 52: 941–952.
- (23) Price JF, Reynolds RM, Mitchell RJ, et al. The Edinburgh Type 2 Diabetes Study: study protocol. *BMC Endocr Disord* 2008; 8: 18.
- (24) Marioni RE, Deary IJ, Murray GD, et al. Genetic associations between fibrinogen and cognitive performance in three Scottish cohorts. *Behav Genet* 2011; 41: 691–699.
- (25) Undas A, Kolarz M, Kopec G, et al. Altered fibrin clot properties in patients on long-term haemodialysis: relation to cardiovascular mortality. *Nephrol Dial Transplant* 2008; 23: 2010–2015.
- (26) Collet JP, Allali Y, Lesty C, et al. Altered fibrin architecture is associated with hypofibrinolysis and premature coronary atherothrombosis. *Arterioscler Thromb Vasc Biol* 2006; 26: 2567–2573.
- (27) Dunn EJ, Ariens RA, Grant PJ. The influence of type 2 diabetes on fibrin structure and function. *Diabetologia* 2005; 48: 1198–1206.
- (28) Henschen-Edman AH. Fibrinogen non-inherited heterogeneity and its relationship to function in health and disease. *Ann N Y Acad Sci* 2001; 936: 580–593.

Table 1. Clinical characteristics and protein levels in carriers of B β Arg448Lys variants of fibrinogen. The B β 448Arg group consists of homozygotes for the B β 448Arg fibrinogen variant. The B β 448Lys group consists of homozygotes for the B β 448Lys fibrinogen variant plus all heterozygotes. SD, standard deviation; T2DM, type 2 diabetes; ACEi, angiotensin-converting-enzyme inhibitor; ARB, angiotensin receptor blocker.

Figure 1. B β Arg448Lys genetic variants of fibrinogen and plasma fibrin networks. The B β 448Arg group consists of homozygotes for the B β 448Arg fibrinogen variant. The B β 448Lys group consists of homozygotes for the B β 448Lys fibrinogen variant plus all heterozygotes. The following clot structure parameters are shown: (A) Lag time; no significant difference with B β 448 allele (B) Lysis time; significant increase with B β 448Lys variant and (C) Maximum absorbance; significant increase with B β 448Lys variant. Data presented are mean \pm SD.

Figure 2. B β Arg448Lys genetic variants and fibrin clots prepared from purified fibrinogen. (A) Clot maximum absorbance; significant decrease with increasing copies of the 448Lys allele. (B) Lysis time; prolongation with increasing copies of the 448Lys allele. Data presented are mean \pm SD.

Figure 3. Visualisation of fibrin clots prepared from B β Arg448Lys genetic variants of fibrinogen using purified protein. (A) Laser scanning confocal microscopy (magnification x4000), showing more compact clots with increasing copies of B β 448Lys allele. Scale bars represent 50 μ M. (B) Scanning electron micrographs (magnification x30000), demonstrating differences in clot structure with the genetic variants of fibrinogen. Scale bars represent 3 μ M. (C) Fibre thickness showing a decrease with increasing copies of B β 448Lys allele. Data presented are mean \pm SD.

Figure 4. B β Arg448Lys fibrinogen variants and age in individuals with type 2 diabetes.

The B β 448Arg group consists of homozygotes for the B β 448Arg fibrinogen variant. The B β 448Lys group consists of homozygotes for the B β 448Lys fibrinogen variant plus all heterozygotes. (A) Patients with the B β 448Lys variant were significantly younger than those with the B β 448Arg variant. (B) When split for gender, the age difference comparing the two genetic variants of fibrinogen significance was only relevant in women. Data presented are mean \pm SD.

Figure 5. Predictive value of B β Arg448Lys fibrinogen variants for cerebrovascular

disease. The B β 448Arg group consists of homozygotes for the B β 448Arg fibrinogen variant. The B β 448Lys group consists of homozygotes for the B β 448Lys fibrinogen variant plus all heterozygotes. B β 448Lys fibrinogen was associated with a history of cerebrovascular disease in the whole group, which was evident in women only after repeating the analysis by gender. Model adjusted for cardiovascular risk factors including total and high density lipoprotein cholesterol, HbA1c, systolic and diastolic blood pressure and smoking. Data displayed as OR \pm 95% CI. OR, odds ratio.

Table I

Variable	Bβ448Arg (n=564)	Bβ448Lys (n=258)	p-value
Age, years (range)	68.3 (60–75)	67.6 (60–75)	0.02
Duration of T2DM, years (±SD)	8.05 (6.57)	8.21 (6.32)	0.78
Current smokers, n (%)	71 (12.6)	36 (14.0)	0.67
Males, n (%)	293 (52.0)	129 (50.0)	0.60
Vascular parameters			
Systolic blood pressure, mmHg (±SD)	133.6 (16.6)	132.2 (15.80)	0.21
Diastolic blood pressure, mmHg (±SD)	69.0 (8.9)	68.5 (8.6)	0.43
Anthropometrics			
Body-mass index, kg/m ² (±SD)	31.0 (5.4)	31.9 (5.8)	0.28
Waist circumference, cm (±SD)	106.3 (13.0)	107.6 (12.5)	0.15
Waist/hip ratio (±SD)	0.96 (0.08)	0.96 (0.07)	0.77
Metabolic and renal			
HbA1c, %/ mmol/mol, (±SD)	7.4 (1.16)/ 57 (134)	7.4 (1.02)/ 57 (11)	0.90
Plasma glucose, mmol/l (±SD)	7.48 (2.03)	7.62 (1.93)	0.34
Total cholesterol, mmol/l (±SD)	4.3 (0.88)	4.3 (0.9)	0.76
High-density lipoprotein cholesterol, mmol/l (±SD)	1.3 (0.35)	1.3 (0.36)	0.37
Estimated glomerular filtration rate, ml/min/m ² (±SD)	64.4 (14.49)	63.6 (14.4)	0.44

Treatment,			
Metformin, n (%)	344 (61.0)	142 (55.0)	0.48
Aspirin, n (%)	364 (64.5)	171 (66.3)	0.82
Insulin, n (%)	57 (10.1)	33 (12.8)	0.31
Thiazolidinediones, n (%)	93 (16.5)	45 (17.4)	0.78
ACEi & ARB, n (%)	383 (67.9)	174 (67.4)	0.95
Statins, n (%)	477 (84.6)	211 (81.8)	0.76
Sulfonylureas, n (%)	180 (31.9)	69 (26.7)	0.27
Nitrates, n (%)	99 (17.6)	44 (17.1)	0.88